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Characterization of new RNA polymerase III and RNA polymerase II transcriptional promoters in the Bovine Leukemia Virus genome

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Bovine leukemia virus latency is a viral strategy used to escape from the host immune system and contribute to tumor development. However, a highly expressed BLV micro-RNA cluster has been reported, suggesting that the BLV silencing is not complete. Here, we demonstrate the *in vivo* recruitment of RNA polymerase III to the BLV miRNA cluster both in BLV-latently infected cell lines and in ovine BLV-infected primary cells, through a canonical type 2 RNAPIII promoter. Moreover, by RPC6-knockdown, we showed a direct functional link between RNAPIII transcription and BLV miRNAs expression. Furthermore, both the tumor- and the quiescent-related isoforms of RPC7 subunits were recruited to the miRNA cluster. We showed that the BLV miRNA cluster was enriched in positive epigenetic marks. Interestingly, we demonstrated the *in vivo* recruitment of RNAPII at the 3'/LTR/host genomic junction, associated with positive epigenetic marks. Functionally, we showed that the BLV LTR exhibited a strong antisense promoter activity and identified *cis*-acting elements of an RNAPII-dependent promoter. Finally, we provided evidence for an *in vivo* collision between RNAPIII and RNAPII convergent transcriptions. Our results provide new insights into alternative ways used by BLV to counteract silencing of the viral 5'/LTR promoter.

Bovine leukemia virus (BLV) is a B-lymphotropic oncogenic deltaretrovirus infecting cattle that shares common biological and structural features with the human T-cell leukemia virus I and II (HTLV-I and II) (reviewed in ref. 1,2). In the majority of cases, infection is asymptomatic but 30% of BLV-infected animals will develop a persistent lymphocytosis and less than 5% will progress to B-cell lymphoma or leukemia, termed enzootic bovine leucosis, after a long period of latency characterized by the absence of viral replication^{3–5}. It is widely accepted that BLV latency is a viral strategy used to escape from the host immune response contributing to tumor development⁶. Remarkably, BLV can be experimentally inoculated into sheep that always develop leukemia or lymphoma after a shorter period of incubation than in cattle, and therefore sheep represent a model to study tumor development^{7,8}.

Transcription of BLV genes initiates at the U3/R junction in the 5'-long terminal repeat (LTR) and is regulated by cellular transcription factors for which several binding sites have been identified in the LTR^{9–19}, by the viral transactivator TAX_{BLV}²⁰ and by the chromatin status of the BLV provirus^{21–25}. Indeed, we have previously demonstrated that the 5'/LTR RNA polymerase II-driven transcriptional repression is due to the epigenetic state of the 5' LTR characterized by weak histone acetylation and DNA CpG hypermethylation associated to closed chromatin in a lymphoma-derived BLV-infected L267 ovine cell line harboring a fully competent provirus^{19,21,25}.

Recent publications from two independent laboratories have identified, by bioinformatics analysis and deep sequencing, a cluster of 10 micro-RNAs (miRNAs) encoded by the BLV genome^{26,27}. miRNAs are small ~22 nucleotides RNAs implicated into the regulation of a constantly increasing number of cellular processes and expressed by a wide majority of eukaryotes and some DNA viruses (reviewed in ref. 28). Despite the 5'/LTR

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silencing dogma, these micro-RNAs are highly transcribed through a non-canonical process, suggesting that the BLV found an alternative way to express a part of its genome in a latency state likely by using an alternative polymerase²⁹.

In the present report, we investigated the *in vivo* recruitment of the RNAPII and RNAPIII complexes to the BLV genome. We demonstrated the recruitment of a *bona fide* RNA polymerase III at the BLV miRNA cluster through a canonical type 2 RNAPIII promoter similar to the one responsible for transfer RNA (tRNA) transcription. We also established a direct functional link between BLV miRNAs expression and RNAPIII transcriptional activity. Next, we showed that both tumor- and quiescent-related isoforms of RNAPIII were recruited to the miRNA cluster and that the miRNA cluster exhibited a profile of positive epigenetic marks (histone acetylation and DNA hypomethylation). Interestingly, in addition to the RNAPIII recruitment, we demonstrated an RNAPII recruitment at the junction between the 3′LTR and the host genome which was also associated with positive epigenetic marks typical of transcriptionally active promoters. Therefore, we tested the potential promoter activity of the LTR and demonstrated that this region was able to drive transcription in the antisense orientation in the nucleosomal context of episomally replicating constructs through a new RNAPII-dependent promoter. Importantly, ChIP-seq experiments performed in a BLV-infected cell line confirmed the high RNAPIII recruitment to the BLV miRNA cluster and the RNAPII occupancy just downstream of this region, suggesting a collision phenomenon between these two polymerase machineries and stalling of RNAPII.

Results

The RNA polymerase III is recruited to the miRNA cluster *in vivo*. Previous studies have provided evidence that the BLV miRNAs are transcribed by RNA polymerase III^{26,27,30}. Indeed, Kincaid *et al.* have identified by bioinformatics analyses RNAPIII initiation *cis*-elements in the BLV miRNA cluster²⁶. Moreover, they have shown by mutagenesis analysis that the identified *cis*-regulatory elements are functional in the context of 293T cells transformed with plasmids coding for BLV miRNA³⁰. Finally, it has been shown that the use of α -amanitin at a concentration known to specifically inhibit RNAPII but not RNAPIII did not decrease BLV miRNA production^{26,27,30}. However today, the presence of a functional RNAPIII at the BLV miRNA cluster has not been demonstrated yet. Therefore, we decided to investigate the *in vivo* recruitment to the BLV provirus of RNAPIII by performing chromatin immunoprecipitation (ChIP) assays. For this purpose, we prepared chromatin from the latently BLV-infected L267 cell line and immunoprecipitated the largest RNAPIII subunit (RPC1) using a specific antibody. As controls, we performed ChIP assays with a purified IgG to measure the aspecific background and with an antibody directed against the largest RNAPII subunit (RPB1). Next, purified DNA was amplified by real-time quantitative PCR with oligonucleotide primers hybridizing to thirteen specific regions along the BLV genome and its host environment (Fig. 1a). As shown in Fig. 1b, we observed recruitment of RPC1 to the BLV miRNA cluster, thereby providing the first *in vivo* evidence that the BLV miRNAs are transcribed by RNAPIII. As positive controls, we amplified the immunopurified DNA by real-time quantitative PCR using oligonucleotide primers hybridizing in genes encoding the tRNA for the asparagine amino acid and the 5S ribosomal DNA, both known to be RNAPIII-transcribed genes (reviewed in ref. 31). Our results showed that RPC1 was recruited to the tRNA^{Asp} and the 5S rDNA genes, demonstrating the functionality of the α -RPC1 antibody we used (Fig. 1e.g).

Regarding the recruitment of RNAPII, our previous studies using the L267 cell line failed to demonstrate the recruitment of RNAPII to the 5′LTR, that contains the main transcriptional promoter of BLV genes²¹. In the present study, we confirmed these observations and, as a control, we showed the presence of RNAPII to the first exon of the RASA3 gene, the host gene in which the BLV provirus is integrated in the L267 cell line (Fig. 1b). Surprisingly, we observed an important recruitment of RNAPII to the miRNA cluster, in agreement with previously reported ChIP-seq data showing binding of RNAPII near many known RNAPIII genes³². Interestingly, RNAPII was also recruited at the junction between the 3′LTR and the host genome, suggesting that the 3′LTR may also contains an RNAPII-dependent transcriptional promoter as previously described for the related oncogenic deltaretrovirus HTLV-I³³.

In order to further validate our results, additional ChIP experiments were performed in the YR2 cell line, another latently BLV-infected ovine cell line. These cells are considered as a model for defective latency since two E- to K-amino acid substitutions in the viral transactivator TAX_{BLV} impair the infectious potential of the integrated provirus³⁴. Using the YR2 cell line, we observed recruitments of RNAPIII and RNAPII to the BLV genome similar to those observed in the L267 cell line (Supplementary Fig. S1). However, as opposed to what we observed in the L267 cell line, RNAPII was also recruited at the 5′LTR, confirming our previous results obtained in the YR2 cell line²¹ and supporting the deficiency of transactivation mediated by TAX_{BLV} allowing the formation of the RNAPII initiation complex but not an efficient transcriptional elongation^{24,35}.

To confirm our results in a more physiological model of BLV-infected cells, we next evaluated the *in vivo* recruitment of RNAPIII to the BLV provirus in peripheral blood mononuclear cells (PBMCs) isolated from a BLV-infected sheep that developed leukemia. We demonstrated recruitment of RPC1 to the BLV miRNA cluster (Fig. 1c), thereby confirming, in BLV-infected ovine primary cells, the data we obtained in cell lines. These latter results also validated the L267 and YR2 cell lines as an appropriate model to further characterize the RNAPIII promoter present at the BLV miRNA cluster.

To further evaluate the *in vivo* RNAPIII recruitment to the BLV miRNA cluster, we assessed the presence of other RNAPIII subunits by ChIP assays. As shown in Fig. 1d, we demonstrated that the RPC1, RPC4 and RPC6 RNAPIII subunits were recruited to the miRNA cluster. Therefore, we formally identified the presence of a *bona fide* RNAPIII transcriptional machinery in the BLV miRNA cluster. As positive controls, we showed recruitment of the different RNAPIII subunits to the tRNA^{Asp} and 5S rDNA genes (Fig. 1e.g).

Finally, as the BLV 5′LTR is known to be regulated by cellular transcription factors, by the viral transactivator TAX and by the cellular activation state, we tested whether the BLV 5′LTR transcriptional activity could modulate the recruitment of RNAPIII and RNAPII to the BLV miRNA cluster and to 3′LTR/host junction. To this end, we

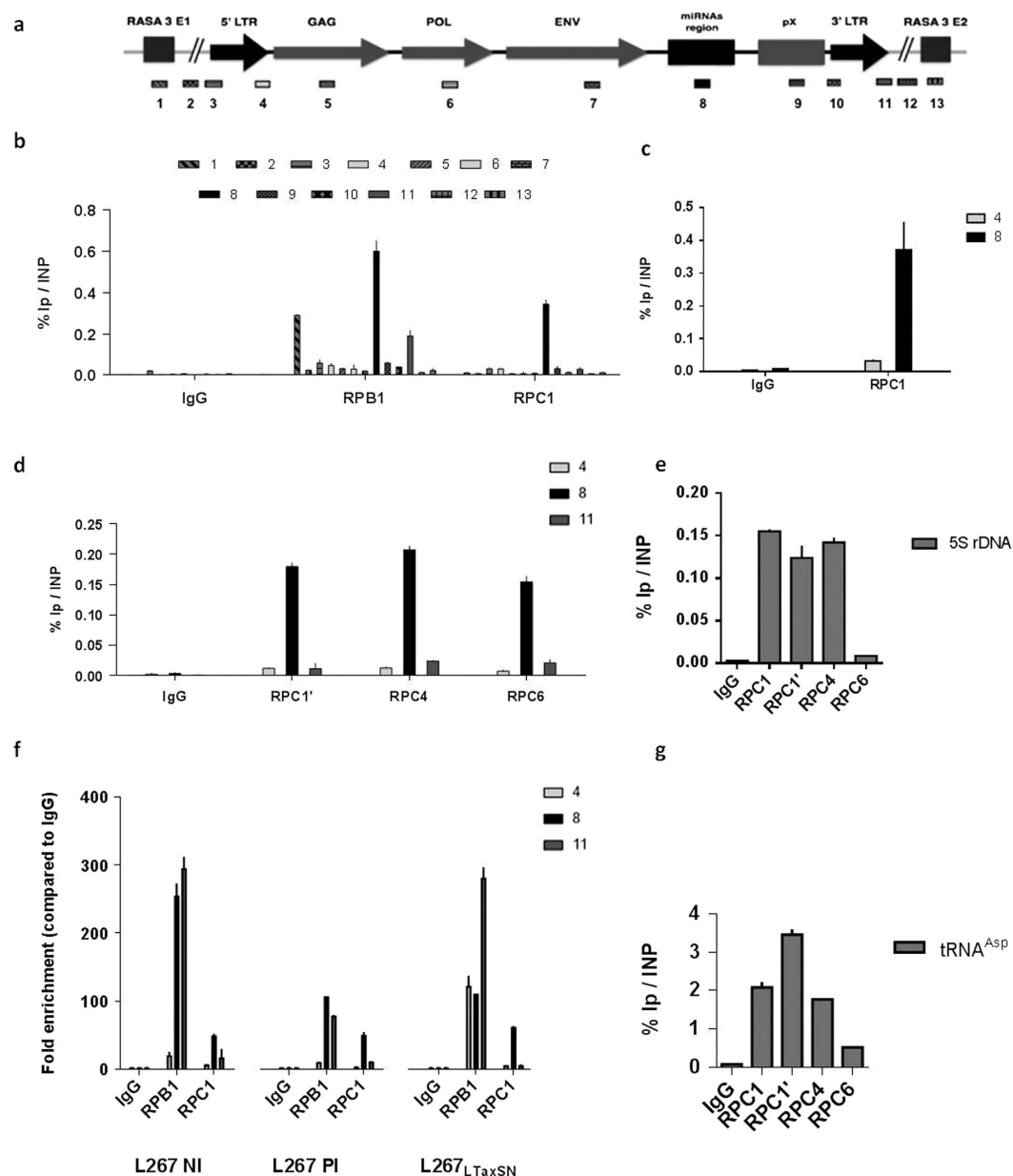


Figure 1. *In vivo* binding of RNAPIII to the BLV miRNA cluster. (a) Schematic representation of the BLV provirus in latently-infected L267 ovine cells. The localization of the different amplified regions is presented with respect to the BLV genome. (b–g) Chromatin prepared from L267 cells (b,d–g), BLV-infected ovine PBMCs (c) or L267_{LTaxSN} cells (f) was immunoprecipitated with specific antibodies directed against different subunits of the RNAPIII as indicated, the largest subunit of the RNAPII or with an IgG as background measurement. Purified DNA was then amplified with oligonucleotide primers hybridizing to either the BLV genome, its host cellular surrounding DNA environment (b–d,f) or to the two class III genes encoding 5S rDNA (e) and tRNA^{Asp} (g). Results are presented as histograms indicating percentages of immunoprecipitated DNA compared to the input DNA (% IP/INP; b–e,g) or indicating fold enrichment above the value obtained with IgG, which was arbitrarily assigned the value of 1 (f). Data are the means \pm SEM from one representative of at least three independent experiments.

carried out ChIP experiments using chromatin from L267_{LTaxSN} cells, a virus-expressing cell line resulting from the transduction of the L267 cell line with a retroviral vector allowing the overexpression of the viral transactivator Tax_{BLV}²³, or from L267 cells treated with a combination of phorbol 12-myristate 13-acetate (PMA) plus ionomycin, one of the most potent activators of BLV genes expression. Since the background levels between the two cell lines were different, recruitment of the different proteins was expressed as fold induction compared to the IgG control. Our results demonstrated that, following induction of the 5'LTR transcriptional activity by the PMA/ionomycin cellular treatment, recruitment of RPC1 to the miRNA cluster was not affected (Fig. 1f). In addition, following transactivation of the 5'LTR by overexpression of TAX_{BLV} in the L267_{LTaxSN} cells, we observed no change

in the recruitment of the RPC1 subunit to the miRNA cluster nor of the RPB1 subunit to the 3'/LTR/host junction (Fig. 1f) compared to the data we obtained in absence of TAX_{BLV} in the L267 cells. As expected, we showed an increase in RNAPII recruitment to the 5'/LTR due to the transactivation by TAX_{BLV}. On the contrary, we observed a large reduction of RNAPII recruitment to the miRNA cluster in both conditions of activation (see Fig. 1f).

Taken together, our results demonstrate that RNAPIII is recruited to the BLV miRNA cluster *in vivo* and is not dependent on the viral 5'/LTR transcriptional state. Moreover, the recruitment of RNAPII occurs at the 3'/LTR/host junction, suggesting the presence of a so far unidentified RNAPII promoter in this region.

BLV miRNAs expression is impaired by depletion of RPC6. Since RNAPIII is recruited *in vivo* to the miRNA cluster, we next investigated a potential functional link between RNAPIII transcriptional machinery and BLV miRNAs expression. With RPC3 and RPC7, the RPC6 subunit has been demonstrated as a member of a subcomplex which is essential for RNAPIII transcriptional initiation but not for RNAPIII transcriptional elongation and termination³⁶. Therefore, we depleted the RNAPIII subunit RPC6 by RNA interference assays using small hairpin RNAs (shRNAs) since RPC6 is the only RNAPIII subunit for which RNA interference experiments have been performed in mammalian cells³⁷.

To avoid off-target effects of the shRNAs, we used two different shRNAs recognizing distinct target sites in the RPC6 mRNA. We first attempted to deplete RPC6 by transfecting or transducing latently BLV-infected L267 and YR2 cell lines. Unfortunately, due to the low transfection or transduction efficiencies in these cell lines, we were unable to show a decrease either in RPC6 protein level or in BLV miRNA transcripts level. Therefore, we performed transduction experiments in the 293T cell line, using lentiviral particles containing shRNAs against RPC6 or a non-targeting shRNA as control. Transduced cells were then transfected with a plasmid expressing the entire miRNA cluster (pmiR). As an internal control for transfection efficiency, cells were cotransfected with a plasmid expressing the *Firefly* luciferase under the control of the Herpes Simplex Virus thymidine kinase promoter (pTK-luc). Forty-eight hours post-transfection, cells were harvested and lysed. Total RNA and protein extracts were used in RT-qPCR and western blot experiments to measure BLV miRNAs expression levels and RPC6 protein levels, respectively.

Our results showed that, despite the absence of cellular toxicity as evaluated by protein quantification, the level of RPC6 decreased by 76% and 77%, respectively, after transduction of the two shRNA against RPC6, in comparison with the level after transduction of the non-targeting control shRNA (Fig. 2a). Moreover, we showed that both shRNAs directed against the RNAPIII-specific RPC6 subunit decreased expression of BLV-miR-B4-3p (Fig. 2b) and BLV-miR-B2-5p (Fig. 2c), the two most abundant viral miRNAs²⁷. These results demonstrate, for the first time, a direct functional link between RNAPIII transcriptional activity and BLV miRNAs expression. As controls, we showed (i) that the non-targeting shRNA did not affect BLV miRNAs expression (Fig. 2b,c), (ii) that the level of the cellular miR-191 (an RNAPII-transcribed miRNA³⁸) was not decreased following RPC6 knock-down (Fig. 2d) and (iii) that transfection efficiencies were similar between the different conditions as shown by the stability of *Firefly* luciferase mRNA level (Fig. 2e).

Thus, our results show that BLV miRNAs expression is impaired following knock-downed expression of RPC6, a member of a subcomplex essential for RNAPIII transcriptional initiation.

The BLV miRNAs are transcribed from a type 2 RNAPIII promoter. Three different types of RNAPIII promoters are known according to the nature and position of specific *cis*-regulating elements that allow recruitment of specific RNAPIII transcription initiation factors (reviewed in ref. 31,39,40). Interestingly, each type of promoter is responsible for the transcription of different class III genes: type 1, 2 and 3 promoters are typical of 5S rDNA, tRNAs and small nuclear and nucleolar (i.e. U6 RNA and 7SK RNA) genes, respectively.

To further physically characterize the BLV RNAPIII promoter responsible for BLV miRNAs transcription, we assessed the promoter type using ChIP experiments. For this purpose, sonicated chromatin from L267 cells was immunoprecipitated with specific antibodies directed against various RNAPIII transcription initiation factors, or with a purified IgG to measure aspecific background. As shown in Fig. 3a, transcription initiation factors specific for RNA polymerase III were present in the miRNA cluster region of the BLV provirus. More specifically, we did not observe recruitment of TFIIB, a factor known to be associated only with the type 1 RNAPIII promoter. Moreover, we demonstrated the recruitment of TFIIC, a factor present in both type 1 and type 2 RNAPIII promoters³¹. In addition, we showed that the TFIIB complex present at the BLV miRNA cluster was composed of the Bdp1 and Brf1 subunits in association with TBP, while the Brf2 subunit, specific of the type 3 RNAPIII promoters³¹, was absent (Fig. 3a).

In order to validate the specificity of the different antibodies, we amplified immunopurified DNA by real-time quantitative PCR with oligonucleotide primers hybridizing to the 5S rDNA gene containing a type 1 RNAPIII promoter (Fig. 3b), to the tRNA^{Asp} gene containing a type 2 RNAPIII promoter (Fig. 3c) and to the 7SK gene containing a type 3 RNAPIII promoter (Fig. 3d). Our results showed that all RNAPIII transcription initiation factors were present at the corresponding control regions in accordance with their expected localization, thereby validating the specificity of the antibodies used in ChIP experiments.

Taken together, our results demonstrate that the promoter responsible for transcription of the BLV miRNAs is a canonical type 2 RNAPIII promoter, as those driving tRNAs transcription, as previously suggested by the *cis*-regulatory elements identified in the miRNA cluster^{26,30}.

The two RNA polymerase III isoforms are recruited to the BLV miRNA cluster. Following a duplication event in an ancestor of vertebrates, 2 genes (*POLR3G* and *POLR3GL*) have been found to encode two isoforms of RPC7 (RPC7 α and RPC7 β , respectively) defining two different RNAPIII complexes⁴¹. The first complex, containing the RPC7 α subunit, is restricted to undifferentiated ES cells and to tumor cells, while the second, containing the RPC7 β subunit, is ubiquitously expressed and essential for cell growth⁴².

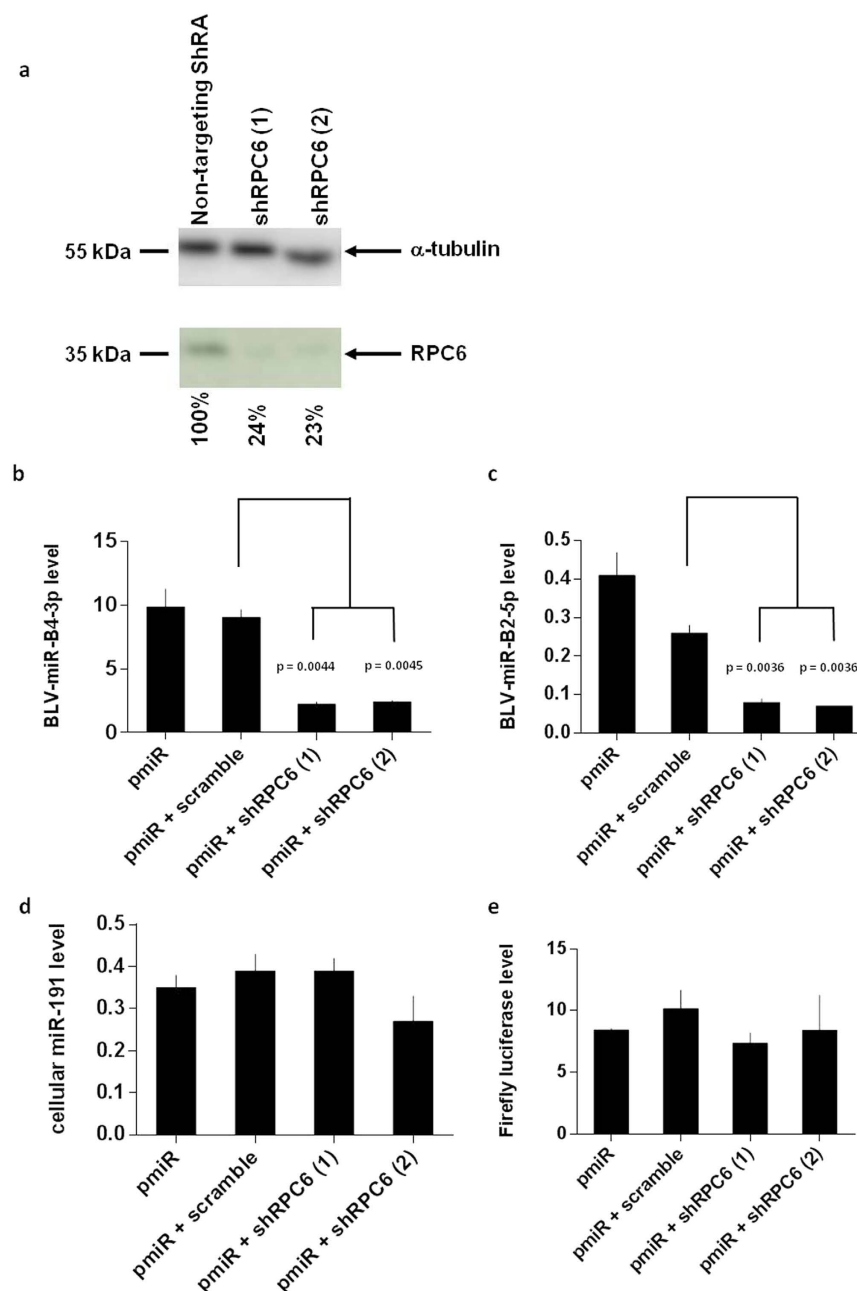


Figure 2. Knockdown of RPC6 induces a decrease in BLV miRNAs expression. The 293T cell line was transduced with lentiviral vectors expressing different shRNAs directed against RPC6 or a non-targeting shRNA and then transiently transfected with a plasmid expressing the miRNA cluster (pmiR). Forty-eight hours post-transfection, total protein and total RNA were extracted. (a) The α -tubulin (upper panel, loading control) and RPC6 (lower panel) were immunodetected by western blotting. Relative quantifications are presented as percentages of RPC6 level in comparison with the non-targeting shRNA. (b–e) Quantitative real-time RT-PCR experiments were performed to measure BLV-miR-B4-3p (b), BLV-miR-B2-5p (c), cellular miR-191 (d) or Firefly luciferase expression (e). Values were normalized using GADPH gene primers. Data are the means \pm SEM from one representative of at least two independent experiments.

In order to investigate whether cell transformation was required for BLV miRNAs transcription, we evaluated the *in vivo* recruitment to the viral miRNA cluster of the two different RNAPIII complexes. To this end, we performed ChIP assays using chromatin obtained from L267 cells and antibodies directed against the RPC7 α and RPC7 β subunits or a purified IgG to measure aspecific immunoprecipitation background. As shown in Fig. 4a, we showed that both RPC7 α and RPC7 β subunits were recruited to the BLV miRNA cluster.

As positive controls, we amplified immunopurified DNA by real-time quantitative PCR with oligonucleotide primers hybridizing in three known RNAPIII-dependent genes (5S rDNA, tRNA^{Asp} and 7SK RNA). As shown in Fig. 4b, both RPC7 α and RPC7 β subunits were recruited to these three genes. Similar experiments were

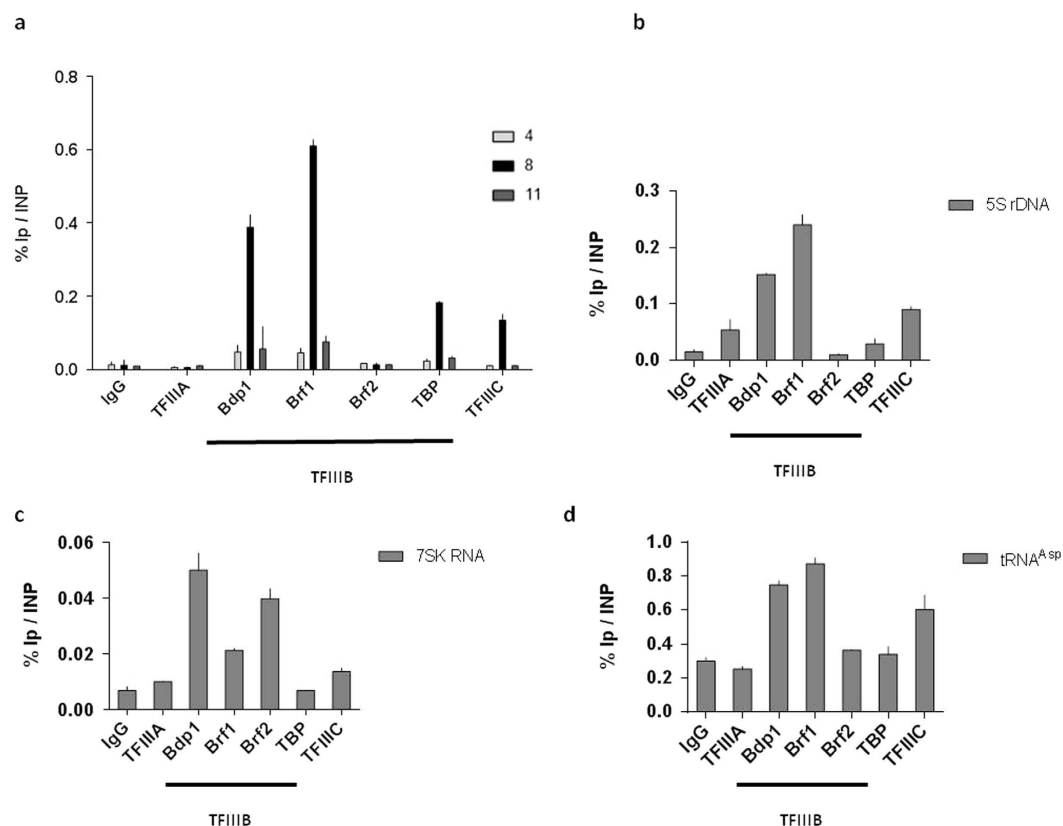


Figure 3. A type 2 RNAPIII promoter drives BLV miRNAs transcription. Chromatin prepared from L267 cells was immunoprecipitated with antibodies directed against different RNAPIII transcription initiation factors or with an IgG as control. Purified DNA was then amplified with oligonucleotide primers hybridizing either to the 5'/LTR, the miRNA cluster or the 3'/LTR/host junction regions (a), either to the type 1 RNAPIII promoter of the 5S rDNA gene (b), the type 2 RNAPIII promoter of the tRNA^{asp} gene (c) or to the type 3 RNAPIII promoter of the 7SK gene (d). Results are presented as histograms indicating percentages of immunoprecipitated DNA compared to the input DNA (% IP/INP). Data are the means \pm SEM from one representative of at least three independent experiments.

performed with sonicated chromatin from the YR2 cell line (Supplementary Fig. S2). Interestingly, as opposed to what we observed in the L267 cell line, only the RPC7 α subunit appeared to be recruited to the BLV miRNA cluster and to control RNAPIII-dependent genes in the YR2 cell line, while both subunits are expressed in YR2 cells (Anne Van den Broeke, personal communication). This unexpected result could possibly be explained by a differential recruitment of RPC7 α and RPC7 β , after cellular transformation, in the L267 cell line compared to the YR2 cell line.

Taken together, our results suggest that the two different isoforms of RNAPIII are able to transcribe the BLV miRNAs at all stages of the BLV disease, in agreement with previous RNA-seq data showing miRNAs expression in transformed and non-transformed cells²⁷.

The BLV miRNA cluster DNA is not methylated. It is known that CpG islands, DNA regions where enriched CpG dinucleotides are observed, may modulate transcriptional activity of a nearby promoter, according with their methylation status⁴³. In addition, our laboratory has previously demonstrated that DNA methylation is an important epigenetic chromatin modification that represses BLV transcription from the 5'/LTR in the true latent L267 cell line²⁵. In order to determine whether DNA methylation modulates BLV miRNAs expression, we first performed *in silico* analyses using three different prediction tools (Methprimer, CpGPlot and CpG Island Searcher) to identify putative CpG islands in the region of the miRNA cluster. The three prediction tools identified a CpG island containing the BLV miRNA cluster and spanning a region from nucleotide (nt) + 6342 to nt + 6833 (nt + 1 defined as the first nucleotide of the 5'/LTR after retrotranscription). Then, we evaluated the DNA methylation status of each CpG dinucleotide of the BLV miRNA cluster in the context of integrated proviruses present in L267 and YR2 cells by treating genomic DNA with sodium bisulfite. Our results showed that, except for the third and the fourth CpG located upstream of the first BLV pre-miR, the miRNA cluster was not methylated in both the L267 (Fig. 5) and YR2 (Supplementary Fig. S3) cell lines.

In conclusion, our results indicate that the BLV miRNA cluster exhibits an hypomethylated CpG profile, in agreement with the high expression level of the BLV miRNAs²⁷.

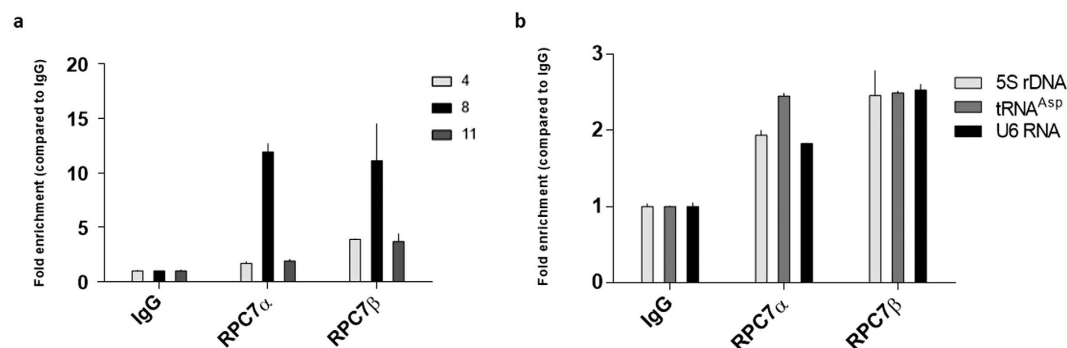


Figure 4. Recruitment of the RPC7 α and RPC7 β subunits to the BLV miRNA cluster. Chromatin prepared from L267 cells was immunoprecipitated with specific antibodies directed against the two isoforms of the RPC7 subunit or with an IgG as background measurement. Purified DNA was then amplified with oligonucleotide primers hybridizing to the 5'LTR, the miRNA cluster or the 3'LTR/host junction regions (a) or to class III control genes (b). Results are presented as histograms indicating fold enrichments compared to the value obtained with IgG to which a value of 1 was assigned. Data are the means \pm SEM from one representative of at least three independent experiments.

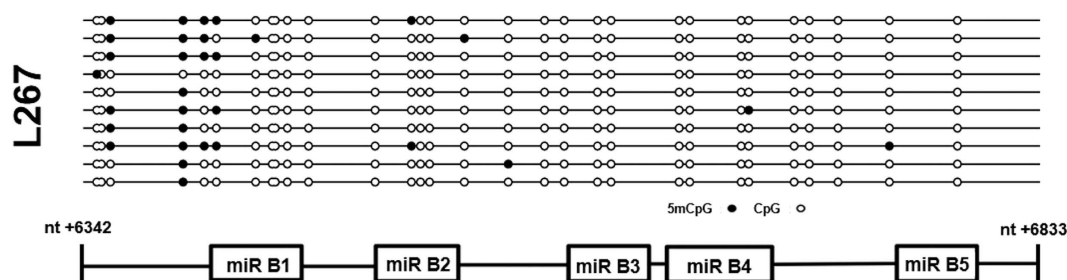


Figure 5. Evaluation of the CpG dinucleotide methylation status of the BLV miRNA cluster. Genomic DNA from L267 cells was extracted and treated with sodium bisulfite and the miRNA cluster was amplified by nested PCR. These amplified products were cloned in a TA cloning vector and 10 independent clones were sequenced. Open and filled circles represent non-methylated and methylated CpG dinucleotides, respectively. The position of the 5 BLV-pre-miRs with respect to the miRNA cluster is presented in the lower part of the figure.

Epigenetic marks associated with active promoters are present to the miRNA cluster and 3'LTR/host cellular DNA junction. In order to further characterize the epigenetic environment present at the BLV miRNA cluster and 3'LTR/host cellular DNA junction, we performed ChIP assays using chromatin from L267 cells and antibodies directed against histone post-translational modifications or the H2A.Z histone variant. As shown in Fig. 6a, we observed the presence, at the level of the miRNA cluster, of acetylated histone H3 (H3Ac), acetylated histone H3 lysine 9 (H3K9Ac) and trimethylated lysine 4 histone H3 (H3K4me3), three chromatin marks associated with RNAPII- and RNAPIII active promoters^{44,45}. Interestingly, those marks were also present at the 3'LTR/host cellular DNA junction, further supporting the presence of an active promoter in this region. In agreement with these results, we did not detect H3K9me3 and H3K27me3, two repressive epigenetic marks associated with transcriptional silencing. As positive controls, we showed the presence of these two repressive marks at the *ZNF12* and *HOXD10* promoter regions (Fig. 6b) as previously reported^{46,47}.

Interestingly, our results showed the presence of H3K4me2 and the histone H2A.Z variant, two epigenetic marks associated with active promoters and enhancers⁴⁵ in the three tested regions (Fig. 6a,c). Moreover, we observed the presence in the three regions of H3K36me3, a positive epigenetic mark associated with RNAPII transcriptional elongation. Remarkably, since the level of H3K36me3 has been previously reported to increase within gene bodies⁴⁵ and since the profile of H3K36me3 shown here was increasing from the 3'LTR to the 5'LTR, our results were in agreement with the presence of an antisense transcription starting from the 3'LTR.

Together, our data strongly support the presence of active promoters at the BLV miRNA cluster and at the junction between the 3'LTR and the host genomic region.

The region of RNAPII recruitment in the BLV 3'LTR exhibits antisense promoter activity. The *in vivo* recruitment of RNAPII at the 3'LTR/host junction (Fig. 1b) and its epigenetic profile characteristic of an active promoter (Fig. 6a,c) prompted us to test its potential promoter activity in a nucleosomal context. To this end, we decided to use pREP-based episomal reporter constructs containing the EBV replication origin and encoding nuclear antigen EBNA-1. Indeed, several studies have reported that pREP-based episomal constructs display hallmarks of proper chromatin structure when transiently transfected into cells^{48,49}. Thus, we cloned

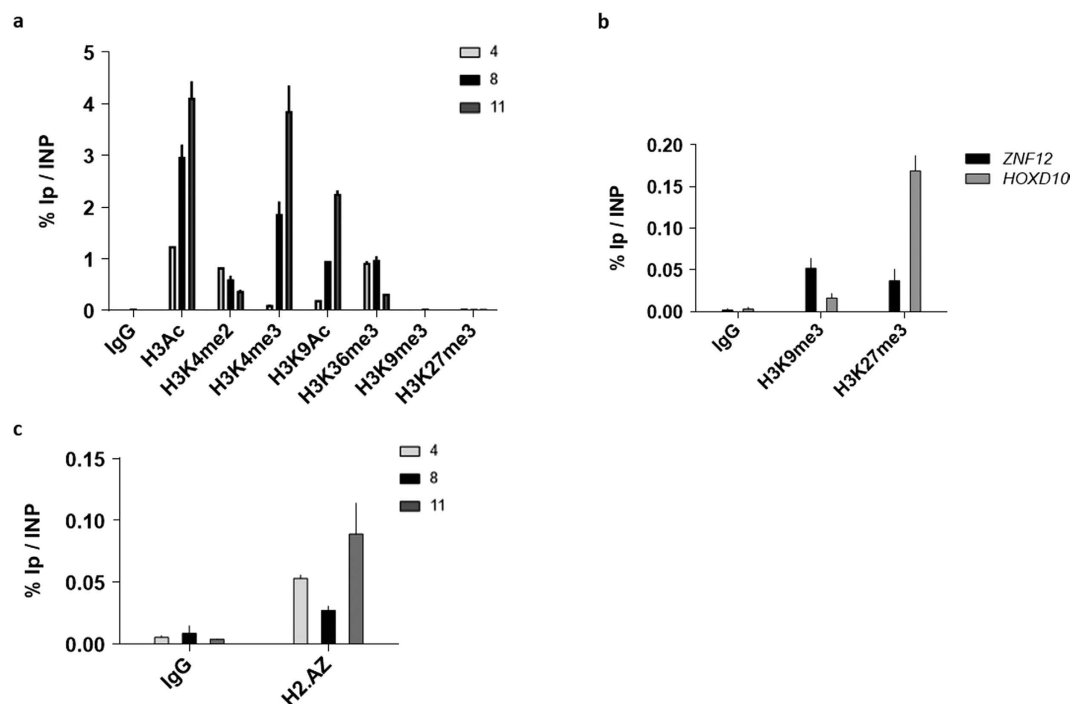


Figure 6. The BLV miRNA cluster and 3′LTR/host genome junction are associated with active epigenetic marks. Chromatin prepared from L267 cells was immunoprecipitated with specific antibodies directed against different histone post-translational modifications (**a,c**), the H2A.Z histone variant (**b**) or with an IgG as background measurement. Purified DNA was then amplified with oligonucleotide primers hybridizing to the 5′LTR, the miRNA cluster or the 3′LTR/host junction regions (**a,c**) or to the promoter regions of the *ZNF12* and *HOXD10* genes (**b**). Results are presented as histograms indicating percentages of immunoprecipitated DNA compared to the input DNA (% IP/INP). Data are the means \pm SEM from one representative of at least three independent experiments.

the complete BLV LTR upstream of the luciferase (luc) gene in both the 5′ and 3′ orientations into a modified pREP10 episomal vector (pREP-luc). The episomally replicating BLV LTR-luc constructs we generated (named pREP-LTR(wt)-S-luc and pREP-LTR(wt)-AS-luc, respectively) were co-transfected into the human B-lymphoid Raji cell line with increasing amounts of a BLV Tax expression vector. Forty-hours post-transfection, cells were lysed and assayed for luciferase activity (Fig. 7a). The pREP-LTR(wt)-AS-luc construct presented a higher (p value = 0.0002) luciferase activity than the pREP-LTR(wt)-S-luc construct, with a 329-fold and 28-fold increase of the luciferase activity, respectively, compared to the parental reporter vector pREP-luc. Moreover, Tax transactivated both the sense and antisense LTR promoter activities (913-fold for the pREP-LTR(wt)-S-luc construct [$p < 0.0001$] and 7.4-fold for the pREP-LTR(wt)-AS-luc construct [$p = 0.0012$]). Therefore, our results are consistent with the fact that the BLV 3′LTR exhibits an antisense promoter activity and that this transcriptional activity is significantly higher ($p = 0.0002$) than the 5′LTR sense promoter activity which is responsible for BLV mRNAs transcription.

To further characterize this newly identified promoter, we performed *in silico* analyses (YAPP Eukaryotic Core Promoter Predictor) of the BLV LTR sequence in the antisense orientation to identify specific *cis*-regulatory elements present in RNAPII-dependent promoters⁵⁰. Our analyses revealed the presence of a putative BRE (nt 29–35, nt 1 defined as the first nucleotide of the 3′LTR in antisense orientation), and overlapping MTE (nt 94–105)/DPE (nt 100–104) motifs, that are both conserved in the nine different BLV genetic groups⁵¹.

In order to functionally characterize these *cis*-regulatory motifs, we introduced individually or in combination point mutations known to abolish the motif's function^{52,53} in the context of the pREP-LTR(wt)-AS-luc construct. The generated plasmids were transfected into the Raji cell line. Forty-hours post-transfection, cells were lysed and assayed for luciferase activity. Results presented in Fig. 7b showed that both mutations in the BRE and MTE/DPE motifs resulted in decreased luciferase activities compared to the promoter activity of the wild type LTR ($p < 0.0001$) suggesting a positive regulatory role of the BRE and MTE/DPE motifs in the BLV LTR antisense RNAPII-dependent promoter activity. Moreover, the combinatory mutation BRE-MTE/DPE resulted in a stronger decrease of the antisense promoter activity ($p < 0.0001$ compared to the reporter vectors containing the individual mutations in BRE and MTE/DPE) supporting the functional role of the identified motifs.

Taken together, our results demonstrate the presence of a transcriptionally active RNAPII promoter in the BLV 3′LTR, associated with positive epigenetic marks and responsible for antisense transcription, a situation reminiscent of the previously reported HTLV-I 3′LTR antisense promoter activity responsible for transcription of the viral *hbx* gene³³.

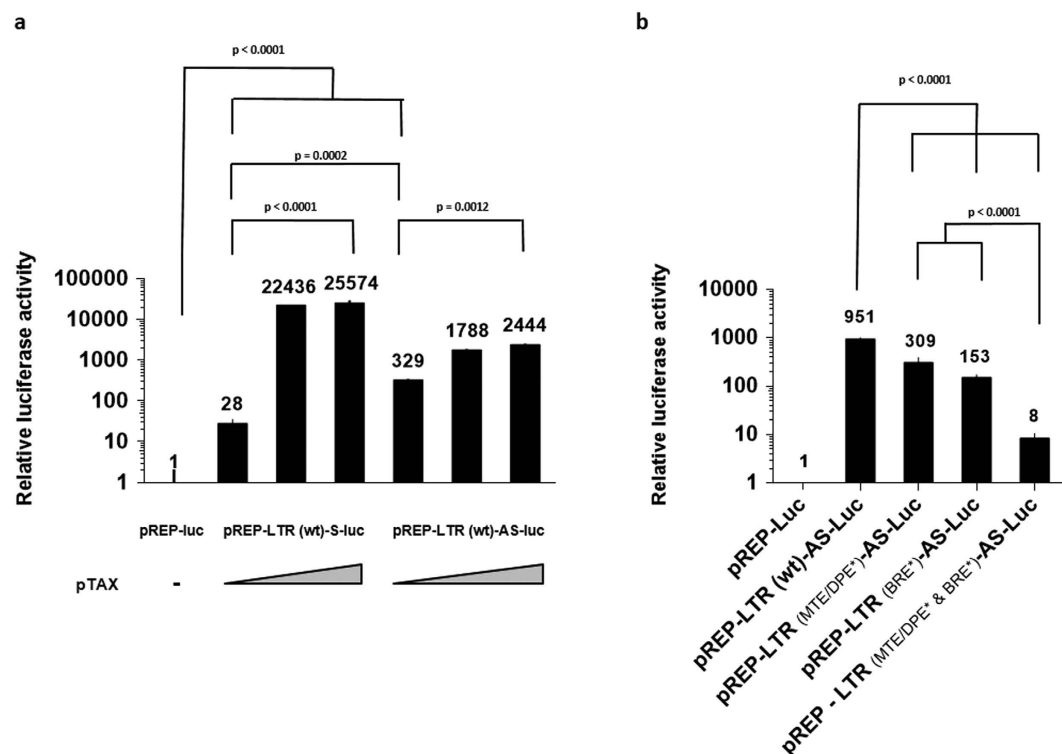


Figure 7. The RNAPII promoter in the 3'/LTR is transcriptionally active. Raji cells were transiently co-transfected with (a) 400 ng of pREP-luc, pREP-LTR(wt)-S-luc or pREP-LTR(wt)-AS-luc constructs, increasing amounts of an expression vector for Tax (50 or 150 ng of plasmid DNA) and 20 ng of pRL-TK or with (b) 400 ng of pREP-luc, pREP-LTR(wt) -AS-luc or pREP-LTR_(MTE/DPE*)-AS-luc, pREP-LTR_(BRE*)-AS-luc or pREP-LTR_(MTE/DPE* & BRE*)-AS-luc mutated constructs and 20 ng of pRL-TK. Forty-eight hours after transfection, cells were collected, lysed and both firefly and renilla luciferase activities were measured. Results are presented as histograms indicating relative luciferase activities compared to the pREP-luc for which a value of 1 was assigned. Data are the mean \pm SEM from one representative of at least three independent experiments.

The RNAPIII collides the RNAPII at the BLV miRNA cluster. As presented in Fig. 1b, we showed an unexpected recruitment of RNAPII to the BLV miRNA cluster. As this presence could be explained by a cooperative binding³² or by the antisense transcription coming from the 3'/LTR (Fig. 7), we decided to investigate the RNAPII and RNAPIII coverage along the BLV genome by ChIP experiments followed by deep sequencing analysis. To this end, sonicated chromatin from L267 cells was immunoprecipitated with specific antibodies directed against the largest subunits of RNAPII (RPB1) and RNAPIII (RPC1) or with a purified IgG as background measurement. The immunoprecipitated DNA was then used for paired-end deep sequencing analysis. Reads were aligned to the ovine reference genome, where BLV sequence has been inserted at the integration site of the L267 cell line. Results of single-matched are presented in Fig. 8a.

As shown in Fig. 8a, we confirmed the presence of both RNAPII and RNAPIII complexes at the BLV miRNA cluster in agreement with our quantitative PCR results (Fig. 1b). Interestingly, our results revealed the presence of RNAPIII at the miRNA cluster, while RNAPII coverage was further downstream. This localization of RNAPII downstream relative to the RNAPIII transcriptional start site contrasted with previous studies showing cooperation between these two polymerases where RNAPII is present upstream the RNAPIII-dependent unit^{44,54}. Therefore, our ChIP-seq data supports the evidence of an antisense RNAPII-dependent transcription initiating in the 3'/LTR. Moreover, there was a drastic drop in RNAPII ChIP-seq coverage at the miRNA cluster downstream of the RNAPIII coverage, further supporting convergent transcriptions and collision between RNAPIII and RNAPII (Fig. 8a). However, no recruitment of RNAPII to the 3'/LTR was observed. This result was due to the low quality of the read alignment in the LTRs and explained by the formation process of these LTRs during the reverse transcription leading to regions with identical nucleotide sequences therefore avoiding unambiguous mapping of the reads. In order to have a better view of the RNAPII coverage at the different LTRs, we performed a new alignment of the reads allowing one or two matches to our reference genome. We decided to focus on unique DNA sequences comprising the regions adjacent to the LTRs boundaries (Fig. 8b). These results revealed a higher coverage of the 3'/LTR/host junction, (22 and 2 reads covering the junction after chromatin immunoprecipitation with RNAPII antibody and IgG, respectively) compared to that of the 5'/LTR/viral genome junction (8 and 5 reads covering the junction after chromatin immunoprecipitation with RNAPII antibody and IgG, respectively). However, we showed that the coverage of the 3'/LTR/viral genome (5 and 0 reads covering the junction after chromatin immunoprecipitation with RNAPII antibody and IgG, respectively) and of the 5'/LTR/host genome (5 and 1 reads covering the junction after chromatin immunoprecipitation with RNAPII antibody and IgG, respectively) were similar.

Altogether, our data are further confirming RNAPII recruitment to the transcriptional promoter located at the end of the 3′LTR (Fig. 8b) and indicate the presence of an active RNAPII-dependent promoter in the BLV 3′LTR, responsible for an antisense transcription and colliding with the RNAPIII transcribing the viral miRNA cluster.

Discussion

One of the major features of BLV infection is the absence of viremia due to the RNA polymerase II 5′LTR-driven transcriptional repression. Nevertheless, recent reports using bioinformatics analysis²⁶ and high throughput sequencing of small RNAs from BLV-infected cells²⁷ have shown the presence of a cluster coding for viral miRNAs located between the end of *env* gene and the pX region. Some evidence support the notion that the miRNA cluster is transcribed by the RNA polymerase III, although a direct functional link between RNAPIII transcriptional activity and BLV miRNAs expression has not been reported to date. Indeed, the algorithm used by Kincaid and coworkers has been designed to find RNAPIII-specific initiation *cis*-regulatory elements that have been functionally characterized³⁰, in the genome of retroviruses and this analysis has identified BLV as the only retrovirus containing such *cis*-regulatory elements²⁶. Moreover, α -amanitin treatment at a concentration known to inhibit specifically RNAPII but not RNAPIII transcriptional elongation⁵⁵ did not affect BLV miRNA expression^{26,27,30}. In the present report, we aimed at providing a direct demonstration that the RNAPIII is responsible for BLV miRNA transcription and is recruited to the viral miRNA cluster *in vivo*, and at epigenetically and functionally characterizing its promoter. To this end, we performed chromatin immunoprecipitation assays in two BLV latently-infected ovine cell lines and in BLV-infected primary B cells.

We firstly demonstrated that all tested RNAPIII subunits were recruited to the miRNA cluster *in vivo* (Fig. 1b,d), supporting the presence of a *bona fide* RNAPIII at this genomic region in the context of a complete BLV provirus and confirming previous evidence^{26,27,30}. Furthermore, we also demonstrated the recruitment of the RPC1 RNAPIII subunit in primary PBMCs from a BLV-infected leukemic sheep suffering from leukemia (Fig. 1c), confirming our results in a more physiological model of BLV infection. We also showed that RNAPIII recruitment to the miRNA cluster was not affected by induction of 5′LTR-dependent viral expression either by ectopic expression of Tax_{BLV} (L267_{LTaxSN} cell line) or by PMA/ionomycin treatment (Fig. 1f). These data suggest that the level of 5′LTR transcriptional activity does not seem to influence the recruitment of RNAPIII, in agreement with the stable level of miRNA expression previously observed in L267_{LTaxSN} cells compared to L267 cells²⁷. After confirming the *in vivo* recruitment of RNAPIII to the BLV miRNA cluster, we showed that the viral miRNA expression was significantly decreased upon depletion of RPC6 (Fig. 2b,c), a specific RNAPIII subunit implicated in RNAPIII transcriptional initiation³⁶, functionally demonstrating that the BLV miRNAs are transcribed by RNAPIII. Moreover, two forms of mammalian RNAPIII (α or β) have been identified depending on the presence of RPC7 α or RPC7 β subunit isoforms, respectively^{41,42}. While the RNAPIII β is essential for undifferentiated and differentiated cells, the RNAPIII α is mainly present in undifferentiated and tumor cells, and the level of the RPC7 α mRNA increases during cellular transformation⁴². Interestingly, we showed here that both isoforms of RPC7 were recruited to the miRNA cluster (Fig. 4), suggesting that BLV miRNAs are transcribed by the two forms and do not require cellular transformation. Our results are in agreement with previously reported RNA-seq data²⁷.

Compared to the promoter complexity of RNAPII transcribed genes, the promoter driving RNAPIII transcribed genes is much more simple. Indeed, RNAPIII promoters are composed of a relatively small number of *cis*-acting elements that are generally located within the transcriptional unit, and regulated by a small number of transcription factors (reviewed in ref. 39). In this study, we showed that the TFIIB initiation factor, which is specific of type 1 RNAPIII promoter driving transcription of the 5S rDNA, was not recruited to the BLV miRNA cluster. However, we demonstrated that the TFIIB (composed of TBP, BDP1 and BRF1) and TFIIC complexes were present at the miRNA cluster, supporting the use of a canonical type 2 RNAPIII promoter for BLV miRNA transcription (Fig. 3a) as for cellular tRNA transcription. These results confirm the prediction of the algorithm, based on *cis*-regulatory elements, used by Kincaid and co-workers²⁶.

Our laboratory has previously demonstrated that DNA methylation and histone tail post-translational modifications are important epigenetic chromatin modifications regulating BLV gene expression^{19,21,25}. Indeed, we have previously reported that 5′LTR hypermethylation is associated with the BLV repression in the true latent L267 cell line but not in the defective latency state present in the YR2 cell line²⁵. Here, we showed by bisulfite genome sequencing that the DNA encoding the BLV miRNA cluster, which is located within a CpG island, was not methylated. To further characterize the epigenetic landscape associated with the miRNA cluster, we demonstrated the absence of repressive epigenetic marks (H3K9me3 and H3K27me3) and the presence of epigenetic marks associated with RNAPII- and RNAPIII-dependent active promoters^{44,45}. More specifically, we showed the presence of acetylated histone H3 (H3Ac, H3K9Ac), di- and tri-methylated lysine 4 histone H3 (H3K4me2, H3K4me3). Overall, the activating chromatin environment of the miRNA cluster we demonstrated in the BLV latently-infected cell lines were consistent with the high level of expression of the miRNAs observed in RNA-seq experiments²⁷.

The ChIP results presented here showed an *in vivo* recruitment of RNAPII at the 3′LTR/host junction (Fig. 1b). This specific recruitment was associated with the presence of positive epigenetic marks (such as histone acetylation, H3K4me2, H3K4me3 and the H2A.Z histone variant) and the absence of repressive marks (such as H3K9me3, H3K27me3 (Fig. 6a) or DNA methylation²⁵). Moreover, using episomally replicating luciferase reporter constructs that display the hallmarks of proper chromatin structure when transiently transfected into cells^{48,49}, we demonstrated that the BLV 3′LTR exhibited an antisense promoter activity. BLV 3′LTR antisense promoter activity reported here is further supported by the presence of H3K36me3 along the BLV provirus that is lower at the 3′LTR/host junction than at the miRNA cluster and the 5′LTR. Indeed, H3K36me3 level generally increases along RNAPII transcribed regions⁴⁵, whereas RNAPIII transcribed genes lack for enrichment of this epigenetic mark⁴⁴. In addition, RNA-seq experiments of primary tumors and pre-leukemic clones from BLV infected animals identified the production of two viral antisense transcripts originating in the BLV 3′LTR⁵⁶. Interestingly, the transcriptional start site (TSS) for these antisense transcripts corresponds to the TSS predicted

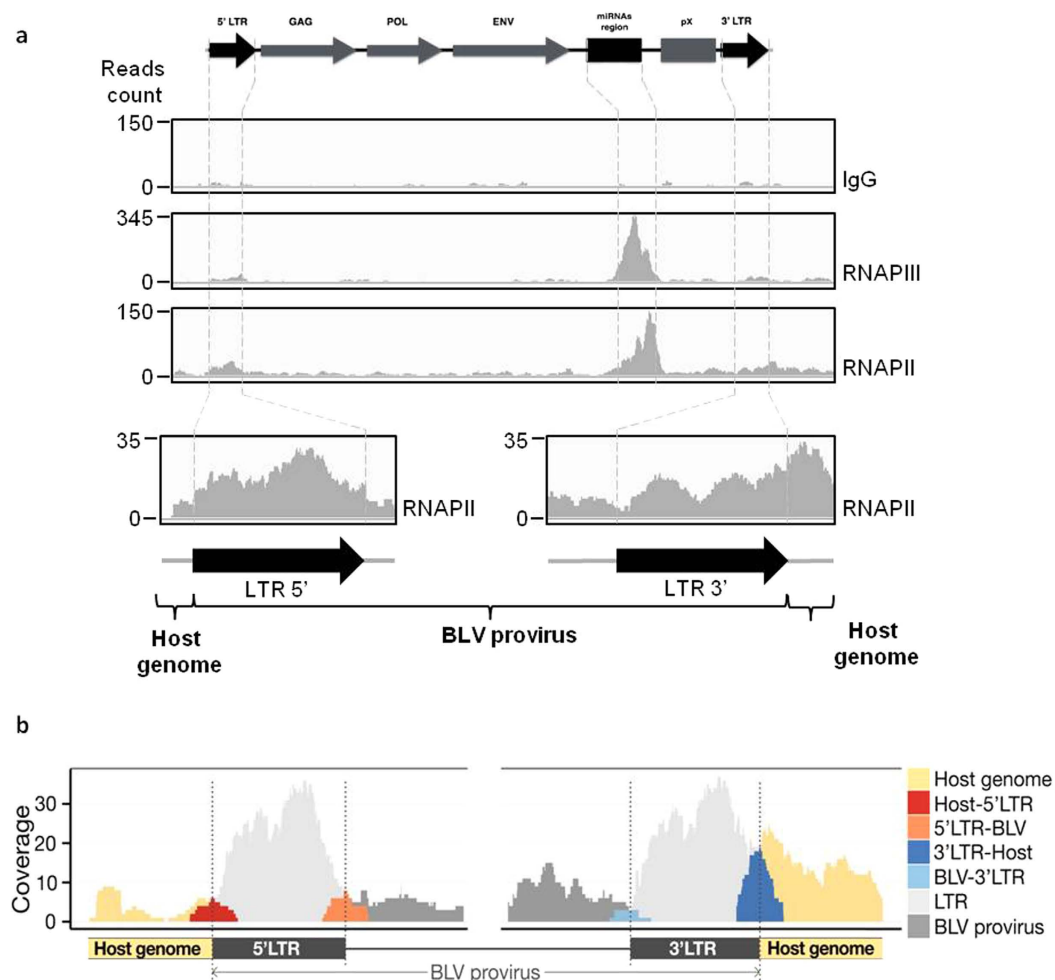


Figure 8. Collision between RNAPIII and RNAPII at the BLV miRNA cluster. View of the tags obtained following ChIP experiments using chromatin prepared from L267 cells and immunoprecipitated with specific antibodies raised against the largest subunit of the RNA polymerase II (RPB1), the largest subunit of the RNA polymerase III (RPC1) or with a purified IgG as background measurement. (a) Single matched reads for RNAPII/RNAPIII coverage along the BLV genome. (b) Zoom-in of the 5' and 3'LTRs regions for single and double matched reads.

by the position of the BRE and MTE/DPE motifs that we identified (Fig. 7b). The identification of a promoter at the end of the BLV 3'LTR could be the counterpart of the previously reported antisense RNAPII promoter responsible for transcription of the *hbx* gene in the closely related HTLV-1 deltaretrovirus³³. Several studies have previously demonstrated the critical role of HBZ expression in HTLV-1-mediated oncogenesis. Indeed, HBZ has been shown to increase HTLV-1-infected T cells proliferation and to inhibit signaling pathways such as the AP-1 and NF- κ B signaling pathways⁵⁷. Moreover, HBZ also contributes to HTLV-1 escape from the host immune system by counteracting Tax functions and therefore by reinforcing viral 5'LTR silencing⁵⁸. In the context of BLV pathogenesis, the antisense transcription originating from this newly discovered promoter could play similar roles in infected B cells proliferation and in the BLV latency. In addition, the antisense transcription could generate small interfering RNAs leading to the cleavage of BLV mRNAs, therefore ensuring the establishment of viral latency and preventing viral reactivation and detection of the infected cells by the host immune system.

It has been shown that α -amanitin treatment, an inhibitor of RNAPII transcriptional elongation when used at low concentration⁵⁵ does not decrease BLV miRNAs expression^{26,27}, thereby indicating that RNAPII is not responsible for the BLV miRNAs transcription. Nevertheless, we showed here the recruitment of RNAPII to the miRNA cluster (Fig. 1b). It is worth noting that Barski and coworkers have previously shown the presence of RNAPII at RNAPIII-transcribed *loci* by ChIP-seq experiments⁴⁴. In addition, it has been proposed that RNAPII activity might facilitate the expression of some RNAPIII products by modifying the local chromatin structure⁵⁴. However, in the context of the BLV provirus, our ChIP-seq experiments suggest that the presence of RNAPII at the miRNA cluster is the result of a collision phenomenon between RNAPIII transcribing the BLV miRNAs in the sense orientation and RNAPII transcribing from the 3'LTR in the antisense orientation (Fig. 8a,b). It has been previously demonstrated *in vitro* that collided RNAPII polymerases resulting from convergent transcription can stop without dissociation from the genomic unit⁵⁹. A similar phenomenon could therefore explain the *in vivo*

RNAPII recruitment we observed here in our ChIP and ChIP-seq experiments (Figs 1b and 8a). Nevertheless, to date, the biological role of convergent transcriptions remains largely unknown. Some studies have shown that antisense transcription could be involved in the masking of splicing sites and thus responsible for the production of different transcript isoforms⁶⁰, whereas other studies have shown that sense and antisense transcriptions can be parts of a self-regulatory circuit allowing a fine-tuned regulation between their respective expression⁶¹. In the context of the BLV provirus, the collision between the two polymerase complexes could affect both RNAPIII and RNAPII transcriptions. Indeed, it has been previously shown that the negative modulation of BLV miRNAs transcription results in an increase of the antisense transcription⁵⁶. Alternatively, it has been shown that the AS1-S harbors a weak termination signal with a canonical polyA sequence, just before the miRNA cluster, but without an adjacent GU-rich consensus⁵⁶. Therefore, the collision between RNAPII transcribing in the antisense orientation from the 3'LTR and RNAPIII transcribing the viral miRNAs could increase the transcription termination efficiency and favor a better release of the AS1-S transcript.

In conclusion, our data demonstrated a direct functional link between RNAPIII transcriptional activity and BLV miRNAs expression, and the *in vivo* recruitment of a *bona fide* RNAPIII complex to the BLV miRNA cluster through a canonical type 2 RNAPIII promoter in BLV-latently infected cell lines and ovine BLV-infected primary cells. Moreover, we demonstrated that the BLV LTR was able to drive transcription in the antisense orientation from a newly identified RNAPII-dependent promoter located at the end of the LTR, as previously reported for the closely related HTLV-1 retrovirus. Importantly, our ChIP and ChIP-seq experiments demonstrated a high RNAPIII recruitment peak in the BLV micro-RNA cluster and an RNAPII recruitment peak just downstream of this region, suggesting a collision phenomenon between the two polymerases and stalling of RNAPII. The two newly identified RNAPIII and RNAPII transcriptional promoters located in the BLV genome brings additional factors in an already complex network of regulators affecting the level of BLV expression and potentially deltaretrovirus induced leukemia.

Methods

Cell lines and cell culture. L267 is a clonal lymphoma-derived B-cell line established from a BLV-infected sheep (S267) injected with naked proviral DNA of an infectious BLV variant⁶², whose provirus displays a wild-type sequence^{23,63}. The L267_{LTaxSN} cell line results from the transduction of native L267 with the pLTaxSN retroviral vector expressing the *tax* cDNA following co-cultivation with the PG13_{LTaxSN} producer cell line and G418 selection of transduced cells^{34,63}. YR2 is a cloned B-lymphoid cell line established from peripheral blood lymphocytes (PBLs) isolated from a BLV-infected sheep⁶⁴, containing a single monoclonally integrated mutated silent provirus^{5,34}. These cell lines were maintained in Opti-MEM medium (Life Technologies) supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM glutamine, non-essential amino acids and 100 µg/ml kanamycin. The 293T cell line (CRL-3216), obtained from American Type Culture Collection (ATCC, Manassas, VA), is established from human embryonic kidney cells that express the large T antigen of simian virus 40 [SV40]⁶⁵. This cell line was maintained in DMEM medium (Life Technologies) supplemented with 10% FBS, 1 mM sodium pyruvate and 1% of penicillin-streptomycin. The Raji cell line⁶⁶, a human B-lymphoid Epstein-Barr virus-positive cell line derived from a Burkitt's lymphoma obtained from the AIDS Research and Reference Reagent Program (National Institute of Allergy and Infectious Disease [NIAID], National Institute of Health [NIH]), was maintained in RPMI 1640-Glutamax I medium (Life Technologies) supplemented with 10% FBS and 1% of penicillin-streptomycin. All the cells were grown at 37 °C in a humidified 95% air/5% CO₂ atmosphere. Frozen primary ovine PBMCs were kindly provided by Anne Van den Broeke (Unit of Animal Genomics, GIGA-R, University of Liège, Belgium).

Plasmid constructs. The eukaryotic expression vectors pTax is a gift from Drs. Luc Willems and Richard Kettmann¹⁵. TRC Lentiviral shRNA plasmids (pLKO.1) against RPC6 and non-targeting control shRNA were purchased from Dharmacon and Sigma-Aldrich, respectively. The pVSG-G and the psPAX2 packaging vectors were obtained from the Reference Reagent Program. To construct the episomally replicating constructs, a fragment containing the luciferase gene and the simian virus 40 (SV40) late poly(A) signal was prepared from pGL3-BASIC (Promega) by digestion with KpnI, blunt-ended with DNA polymerase I large (Klenow) fragment, and digestion with BamHI, successively. This fragment was cloned into pREP10s¹⁹ digested with PvuII and BamHI. The resulting plasmid was designed pREP-luc. A fragment containing the complete LTR of the 344 BLV provirus was isolated from the non episomal pLTRwt-luc¹⁰ by digestion with SmaI. This fragment was cloned in both orientations in the pREP-luc digested with BglII and blunt-ended to obtain the pREP-LTR(wt)-S-luc and pREP-LTR(wt)-AS-luc. The pLTRwt-luc¹⁰ construct was used as a substrate for mutagenesis (by the QuickChange Site-directed Mutagenesis method, Stratagene). Different mutations were generated with pairs of mutagenic oligonucleotide primers (described in Supplementary Table S1). Mutated LTRs were fully sequenced after identification and cloned in pREP10s as described above.

To construct the plasmid expressing the BLV miRNAs, genomic DNA from the L267 cell line was firstly extracted using the DNeasy Blood and Tissue kit (Qiagen) according to the protocol of the manufacturer. The miRNA cluster was then amplified by PCR using specific primers (Supplementary Table S1) and 125 ng of genomic DNA. This PCR reaction was performed at 95 °C for 5 min followed by 35 cycles at 95 °C for 30 sec, 50 °C for 1 min 30 sec and by 60 °C for 7 min. The obtained product was purified on an agarose gel, cloned in the pBS-SKP vector (Promega) and sequenced in order to verify the absence of mutation.

Chromatin Immunoprecipitation assays. ChIP assays were performed as previously described²⁵ using the ChIP assay kit (EMD Millipore). Briefly, cells were cross-linked for 10 min at room temperature with 1% formaldehyde and their chromatin were sonicated (Bioruptor Plus, Diagenode) to obtain DNA fragments of 200–400 bp. Chromatin immunoprecipitations were performed with chromatin from 6 × 10⁶ cells (1 × 10⁶ from the epigenetic modifications) and 5 µg of antibodies (see Supplementary Table S2). Quantitative real-time PCR reactions were performed using 1/60th of the immunoprecipitated DNA and the PerfeCTa SYBRgreen SuperMix

(Quanta BioSciences). Relative quantification using standard curve method was performed for each primer pair and 96-well Optical Reaction plates were read in a StepOnePlus PCR instrument (Applied Biosystem). Fold enrichments were calculated as percentages of input values or as fold inductions relative to the values measured with IgG. Primer sequences used for quantification (see Supplementary Table S3) were designed using the software Primer 3.

ChIP-sequencing assays. ChIP assays were performed as described above. Recovered DNA (~100 ng) was then used for library preparation using the TruSeq ChIP Sample Preparation protocol following Manufacturer's instructions (Illumina Technologies). Paired-end sequencing was then performed with the Illumina HiSeq 2000 instrument. More than 20 millions of single reads were obtained for RPC1 and RPB1 libraries and 10 millions of single reads for IgG library. The single-matched reads were mapped to an hybrid ovine genome (OAR v3.1) containing the BLV provirus sequence (GenBank: KT122858.1) at the L267 integration site (chr10:86299813) using bowtie2 software (-N 1 and -k 5 parameters). Finally, our results were visualized using IGV program. Reads spanning LTR-host and LTR-BLV boundaries were extracted from the alignment file using an in-house R script and ggplot2 was used to plot the ChIP-seq coverage.

Bisulfite-mediated methylcytosine mapping. Genomic DNA was isolated from L267 and YR2 cell lines using the DNeasy Blood and Tissue kit (Qiagen) according to the protocol of the manufacturer. One microgram of genomic DNA was treated with bisulfite with EpiTect Bisulfite kit (Qiagen). The miRNA cluster region of BLV provirus was amplified by two successive PCR reactions (primer sequences are in the Supplementary Table S1). The first PCR reaction was performed at 95 °C for 5 min followed by 35 cycles at 95 °C for 30 sec, 50 °C for 1 min 30 sec and by 60 °C for 7 min. The second PCR reaction was performed at 95 °C for 5 min followed by 40 cycles at 95 °C for 30 sec, 50 °C for 1 min, 72 °C for 1 min 30 sec and by 7 min at 72 °C. The obtained product was purified on an agarose gel and cloned in the pGEM-T easy vector (Promega). At least, 10 clones from each condition were sequenced and analyzed to identify methylated CpGs dinucleotide in this viral region by MethTools software. The frequency of conversion of C to T following sodium bisulfite treatment was greater than 98% at non-CpG sites, indicating the adequacy of the approach.

RNA purification and analysis of transcripts. Total RNA from 3 × 10⁶ cells was extracted using TRIzol (Life Technologies) followed by treatment with turbo DNase (Life Technologies) according to the manufacturer's protocol. Retrotranscription reactions were performed with PrimeScript RT-PCR Kit (TaKaRa) either using 200 ng of RNA and random hexamer oligonucleotides (for luciferase and GAPDH genes) or specific stemloop oligonucleotides for miRNA quantification⁶⁷ (Supplementary Table S1). cDNA was then quantified by real-time PCR as described above (primer sequences are presented in Supplementary Table S3).

Lentiviral production. VSV-G pseudotyped lentiviral particles were produced by transfection of 293T cells (5 × 10⁶/10 cm dish), with the different shRNAs-containing plasmids (9 µg), the pVSV-G (2.25 µg) and the psPAX2 (6.75 µg) packaging vectors by the calcium phosphate transfection method according to the manufacturer's protocol (Promega). 72 hours post-transfection, viral stocks were collected.

Transduction assays. 293T cells (4.5 × 10⁵) were transduced in 6-well plates by 1 mL of undiluted viral stock supplemented by Polybrene at the final concentration of 8 µg/mL. 6 hours post-transduction, the medium was removed and replaced by 2 mL of complete DMEM.

Transient transfection and luciferase assays. 293T cells (4.5 × 10⁵) were transfected with 3 µg of DNA (pmiR and pTK-luc) using FuGENE 6 reagent (Promega) according to the manufacturer's protocol in 6-well plates. Raji cells (3 × 10⁶) were transfected with 750 ng of DNA (pREP-luc constructs, pTAX and pGL4.74 (Promega) as internal control) using the DEAE-dextran procedure as described previously¹⁹. 48 h post-transfection, cells were collected, lysed and luciferase activities were measured. Firefly luciferase values were normalized with respect to the Renilla luciferase values using the DualGlo-luciferase reporter assay system (Promega).

Western blot experiments. Western blotting were performed with 10 µg of the total protein extracts. The immunodetection was assessed using antibodies targeting RPC6 or the α-tubulin (Supplementary Table S2) as loading control. Quantifications of both the RPC6 and the α-tubulin intensities were performed with the ImageJ software (National Institutes of Health, NIH).

Statistical analysis. Data sets from transfection experiments were analyzed using a two-way ANOVA with log-transformed value to ensure homogeneous standard deviation, followed by paired comparisons between samples (Tukey's test). Data sets from RNA quantifications were analyzed using a two-tailed unpaired T test comparisons between samples and the cells transfected with the non-targeting shRNA. The threshold of statistical significance α was set at 0.05. Analyses were performed using Prism version 6.0 (GraphPad software).

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Author Contributions

Conceived and designed the experiments: C.V.L., O.R. and B.V.D. Performed the experiments: B.V.D., A.R., N.D., S.F. and C.V. Analyzed the data: B.V.D., A.R., S.F., A.B., O.R. and C.V.L. Wrote the paper: B.V.D., A.R. and C.V.L.

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